

Sucrose concentration and blueberry plant density in temporary immersion systems (TIS)

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Abstract - The aim was to determine the ideal concentration of sucrose along with the number of plants per container in the face of micropropagation of blueberry under TIS. In the experiment conducted with sucrose concentration (0, 10, 20, e 30 g L⁻¹), we utilized a WPM medium, taking into account 30 explants per container. For the experiment dealing with blueberry plant density, the same medium with 20 g L⁻¹ of sucrose in conjunction with 30, 40, 50 and 60 explants per container was used. After 90 days from the installation of the trial, the following response variables were assessed herein: total length of the plant, number of shoots, length of the largest shoot, number of hyperhydric shoots, length of hyperhydric shoots, and number of healthy leaves. Under a sucrose concentration corresponding to 20 g L⁻¹ the best possible results were obtained. Survival rates of 22 and 80% under the influence of both 10 and 20 g L⁻¹ treatments were reported, pointing out that plants exposed to 20 g L⁻¹ treatment were more amenable to acclimatization. Thus, for the micropropagation of blueberry under TIS the use of 20 g L⁻¹ sucrose and 60 plants/container comes to being highly recommended to increase yield.

Index Terms: *Vaccinium ashei* Reade; Bioreactor, Micropropagation.

Concentração de sacarose e densidade de planta de mirtilo em sistemas de imersão temporária (TIS)

Resumo - O objetivo desta pesquisa foi determinar a concentração ideal de sacarose, juntamente com o número de plantas por recipiente, em face da micropropagação de mirtilo sob TIS. No experimento realizado com concentração de sacarose (0; 10; 20, e 30 g L⁻¹), foi utilizado um meio Woody Plant Medium (WPM), levando-se em consideração 30 explantes por recipiente. Para o experimento de densidade de plantas de mirtilo, foi utilizado o mesmo meio, com 20 g L⁻¹ de sacarose, em conjunto com 30; 40; 50 e 60 explantes por recipiente. Após 90 dias da instalação do ensaio, as seguintes variáveis de resposta foram avaliadas aqui: comprimento total da planta, número de brotos, comprimento da maior brotação, número de brotos hiperídricos, comprimento de brotos hiperídricos e número de folhas saudáveis. Foram relatadas taxas de sobrevivência de 22 e 80% sob a influência dos tratamentos com 10 e 20 g L⁻¹, apontando que as plantas expostas ao tratamento com 20 g L⁻¹ foram mais receptivas à climatização. Assim, a micropropagação de mirtilo sob TIS, 20 g L⁻¹ de sacarose e 60 plantas / recipiente passa a ser altamente recomendada para aumentar a produtividade e também para melhorar a qualidade dos frutos, em pomares em escala comercial.

Termos para indexação: *Vaccinium ashei* Reade; Biorreator, Micropropagação.

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Received: December 24, 2020
Accepted: May 05, 2021

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Introduction

Blueberry (*Vaccinium ashei*) is a shrub belonging to Ericaceae family, which is native from Europe and North America and featuring an eccentric flavor along with nutritional properties that cause the fruit to be highly valued all over the world, with 540 thousand tons produced in 2014 agricultural harvest (CANTUARIAS-AVILÉS et al. 2014). Since its introduction in Brazil, such a woody species has been expanding in terms of planted area; however, this increment is restricted to a low viability of seeds (PASQUALINI et al. 2016). Apart from such a particular problem, further drawbacks related to low quality of plantlets, lack of phytosanitary warranty and delays in production might be circumvented by the micropropagation technique.

This technique improves initial performance of the crop in production fields as opposed to plants grown by means of cuttings (SOUZA et al. 2011, DEBNATH et al. 2012, MARINO et al. 2014). Nevertheless, high *in vitro* atmospheric humidity, low light intensity, high concentration of ethylene (AYUB et al. 2017) along with heterotrophic conditions are conducive to low functionality of the stomata, reduce thickness of the cuticle and also impair photosynthetic system, compromising acclimatization (HAZARIKA 2006, MOHAMED; ALSADON 2010, SILVA et al. 2012).

For blueberry a remarkable alternative lies in the multiplication in bioreactors of temporary immersion systems (TIS), which differently from the conventional system makes use of a liquid medium that provides a more substantial contact between explant and medium in such a way as to better absorption of nutrients and ensure a maximization of crop growth and yield at a given site, apart from allowing for automation of the process by reducing costs plus labor (RODRIGUES et al. 2006, SILVA et al. 2007, TEIXEIRA 2011, GEORGIEV et al. 2014, AYUB et al. 2019).

Therefore, TIS improve the efficiency of the technique by means of air renewal, diminishing gaseous stress and making augmentations at a yield scale quite possible (TEIXEIRA; CID 2014, FLOREZ et al. 2016), leading to reductions in costs with agar and sucrose, since atmospheric air gets into the system (ARAGÓN et al. 2010, RIBEIRO et al. 2016), besides increasing plant density, which in turn culminates in a better reclamation of both space and tissue medium.

In addition to such advantages, TIS allows for entrance of atmospheric air into the system, eliminating toxic gases and also reducing air relative humidity in order to galvanize photosynthesis within an optimal of physiological efficiency. In the face of stimulation of photosynthesis, it is possible to reduce or even eliminate the sucrose used in the tissue medium as a source of carbon (ESCALONA 2003). Some species of blueberry were studied under TIS approach, such as *Vaccinium angustifolium* (DEBNATH, 2009, DEBNATH, 2011) and other species belonging to the groups of highbush, half-high and hybrids (DEBNATH, 2017). Nevertheless, for blueberry pertaining to the Rabbit-eye group, which encompasses species less tolerant to cold, currently there are no research published dealing with protocols established to micropropagation technique under TIS approach.

In view of the aforementioned problem, the aim of the current manuscript was to improve micropropagation of blueberry under TIS by means of the assessment of plant density and ideal sucrose concentration.

Material and methods

Plant Material

Blueberry (*Vaccinium ashei* Reade) previously cultivated *in vitro* at a solid medium WPM (LLOYD; MCCOWN 1980) supplemented with 0.1 g L⁻¹ of myo-inositol, 30 g L⁻¹ of sucrose 6 g L⁻¹ of agar and 5 mg L⁻¹ of 2iP (N6-(2-Isopentenyl) adenine) was used in the experiment. Stakes of 1.5 cm-length along with two buds were employed as explants. The pH was adjusted to 5.0. After 90 days such stakes were utilized for the installation of the trial.

Temporary Immersion Systems - TIS

A doubled-container system with one designed for the explant and another one for a liquid tissue culture featured the TIS under scrutiny, 90 mL of medium at issue was transferred within a 4-hour time period among containers, which remained in the container with explants for 20 seconds. Shortly after culture medium was placed in the container the explants were autoclaved at 120°C for 2 minutes before the installation of the experiment in a laminar flux chamber. The TIS conditions follow the standards reported by Ayub et al. (2019).

Experiment 1: Sucrose concentration

Under TIS conditions, 4 pair of 500 mL-containers were used to test 4 concentrations of sucrose: 0, 10, 20 and 30 g L⁻¹. The liquid medium WPM was supplemented with 0.1 g L⁻¹ of inositol plus sucrose, 5.0 mg L⁻¹ of 2iP and pH adjusted to 5.0. 30 blueberry explants of 1.5 cm-length were added to containers fixated into phenolic foam, having been assessed after 90 days the following response variables: total length of the plant, shoot length, hyperhydric shoot length, total number of shoots, total number of hyperhydric shoots, overall of healthy shoots, total number of leaves, number of hyperhydric leaves, overall number of dead leaves, overall number of healthy leaves, and fresh weight of the plant.

Experiment 2: Plant acclimatization

In order to assess survival throughout acclimatization at 30 days, an experiment set up under a completely randomized 2 x 2 factorial experimental design has been carried out. For such, blueberry plants from plots subjected to treatments of 10 and 20 g L⁻¹ of sucrose encompassing a bigger number of plants amenable to acclimatization along with two distinct sites for it were taken into account in the current study. An acclimatization chamber with relative humidity fluctuations ranging from 75 to 90% and glasses covered with plastics conditioned to an air relative humidity threshold above 90% turned out to be the controlled environment under which the experiment has been conducted with four replications for each treatment adopted herein (Figure 1).

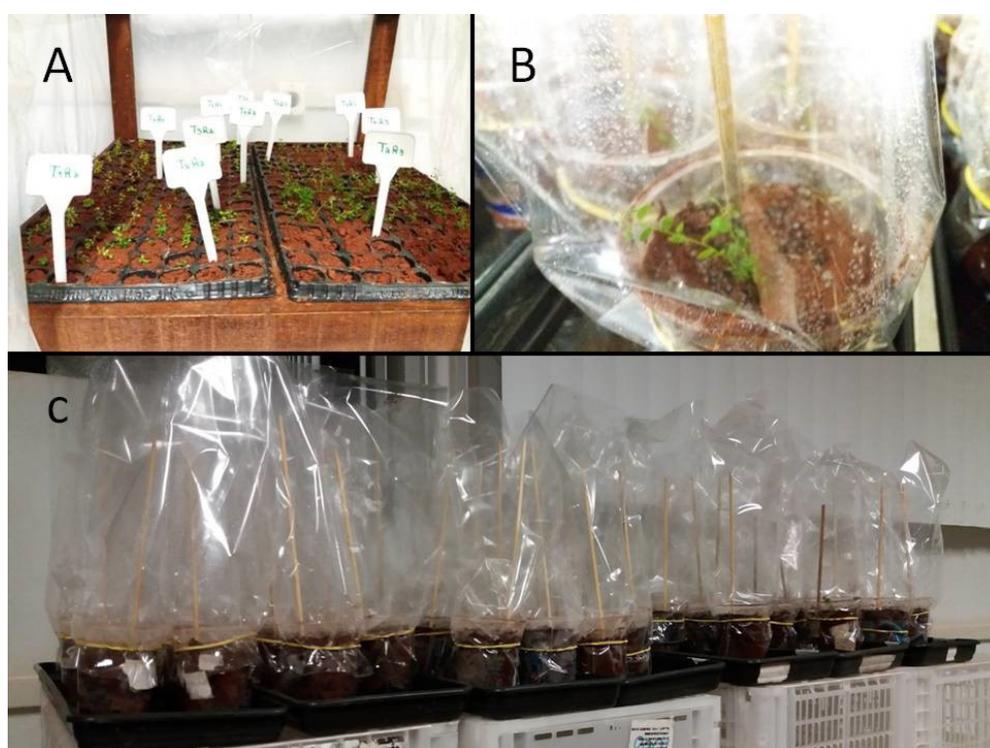


Figure 1. Acclimatization experiment of blueberry. A - Acclimatization chamber. B - plastic cup.

Experiment 3: Plant density

In light of TIS approach, four pair of containers were used to test four plant densities: 30, 40, 50 and 60. The liquid medium (WPM) was supplemented with 0.1 g L⁻¹ of inositol, 30 g L⁻¹ of sucrose, 5.0 mg L⁻¹ of 2iP and pH adjusted to 5.0. In each single container (500-mL capacity) blueberry explants of 1.5 cm-length were fixated in phenolic foam and after 90 days the following response variables were evaluated: total length of the plant, shoot length, total number of shoots, total number of leaves, overall number of dead leaves, fresh weight of the plant, and number of acclimatized plants.

Growth room

The experiments were conducted at a growth room set up to 25 ± 2 °C-air temperature, 16-hour photoperiod and a photon flux density corresponding to 27 μmol m⁻² s⁻¹.

Statistic experimental design

The experiments were carried out under completely randomized experimental design, taking into consideration four replications. Experimental data were submitted to analysis of variance (ANOVA) along with application of Shapiro Wilk and Tukey tests, as well as to a polynomial regression study by means of the statistical program Sisvar (FERREIRA, 2014). The maximum point was calculated as a function of the derivate of the regression equations obtained.

Results and discussion

The best growth performance of the plants under TIS approach has been obtained at a concentration of 15 g L⁻¹ of sucrose, in compliance with the inflexion point of the regression curve (Figures 2, 3, 4, and 5). (Fig. 2:

$\hat{Y}_{\text{Total length}} = 17.357 + 2.1986X - 0.072X^2$). However, under the influence of such a treatment it was possible to notice a more pronounced incidence of hyperhydricity (10 g L⁻¹) (Figures 6 A, B) on both shoots and leaves, which comes to being quite undesirable.

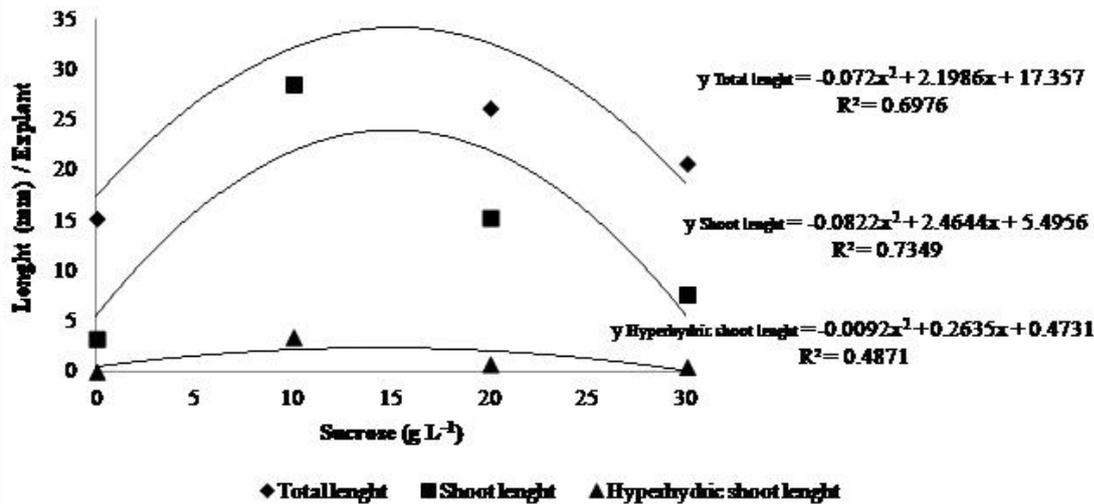


Figure 2. Influence of sucrose on plant total length, shoot length and hyperhydric shoot length of blueberry plants cultivated under TIS. Correct on all models.

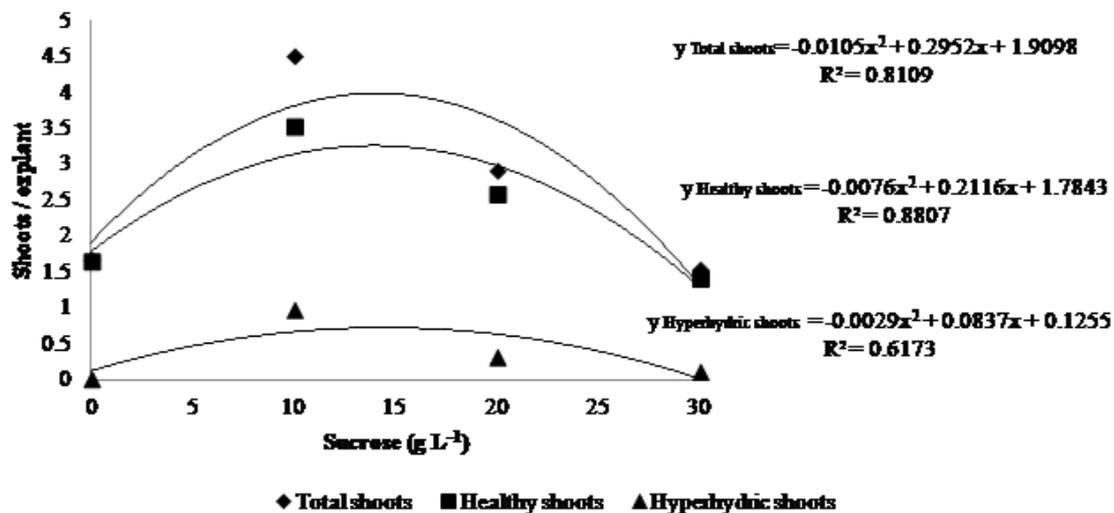


Figure 3. Influence of sucrose on the number of total shoots, healthy shoots and hyperhydric shoots per explant of blueberry cultivated under TIS.

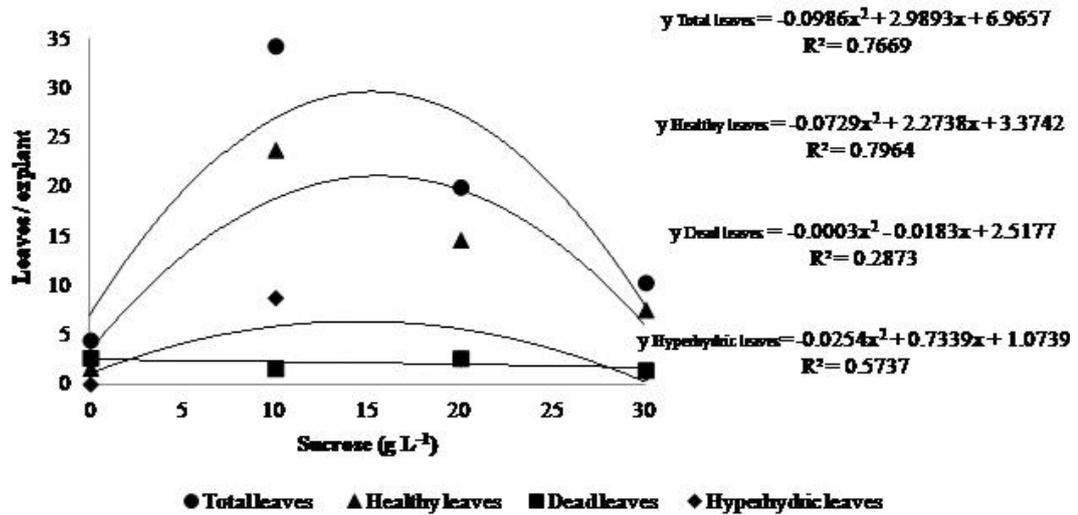


Figure 4. Influence of sucrose on the total amount of leaves, healthy leaves, dead leaves and hyperhydric leaves per explant of blueberry cultivated under TIS.

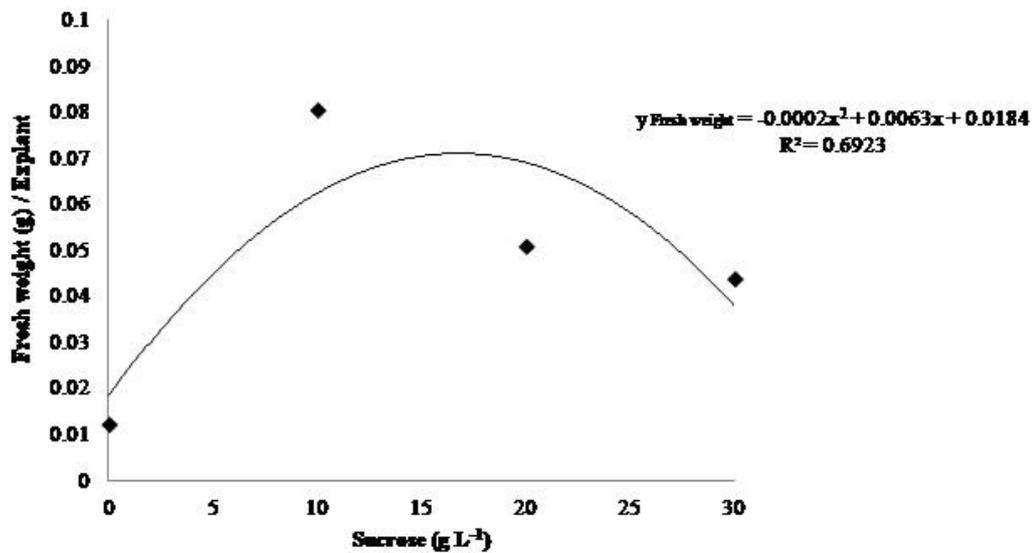


Figure 5. Sucrose influence on blueberry fresh weight per explant cultivated under TIS.

Throughout acclimatization, we observed that in view of variable air relative humidity thresholds the 10 g L⁻¹ of sucrose treatment was conducive to a 22% survival, whereas blueberry plants exposed to the 20 g L⁻¹ of sucrose treatment resulted in an 80% survival rate (Figure 7). Conversely, acclimatized plants at constant relative humidity levels did not show statistical differences among different concentrations of sucrose (Figure 7).

At the plant density experiment, statistical discrepancies were not evidenced for the following response variables: total length of the plant (18.73 mm), number of shoots (1.73), shoot length (9.4 mm), total number of leaves (9.63), overall number of dead leaves (1.1) for each single explant. However, with regards to fresh mass and acclimatization survival the experimental data better fitted a quadratic function in statistical terms.

Increases in total number of plants caused fresh mass per explant to reduce up to 51.66 plantlets per container (Figure 8A), as well as survival percentage to drop up to 57.11 plantlets per container (Figure 8B). From such a point on, rises in overall number of plants favored augmentation in fresh mass of the plant and also in survival rates throughout acclimatization.

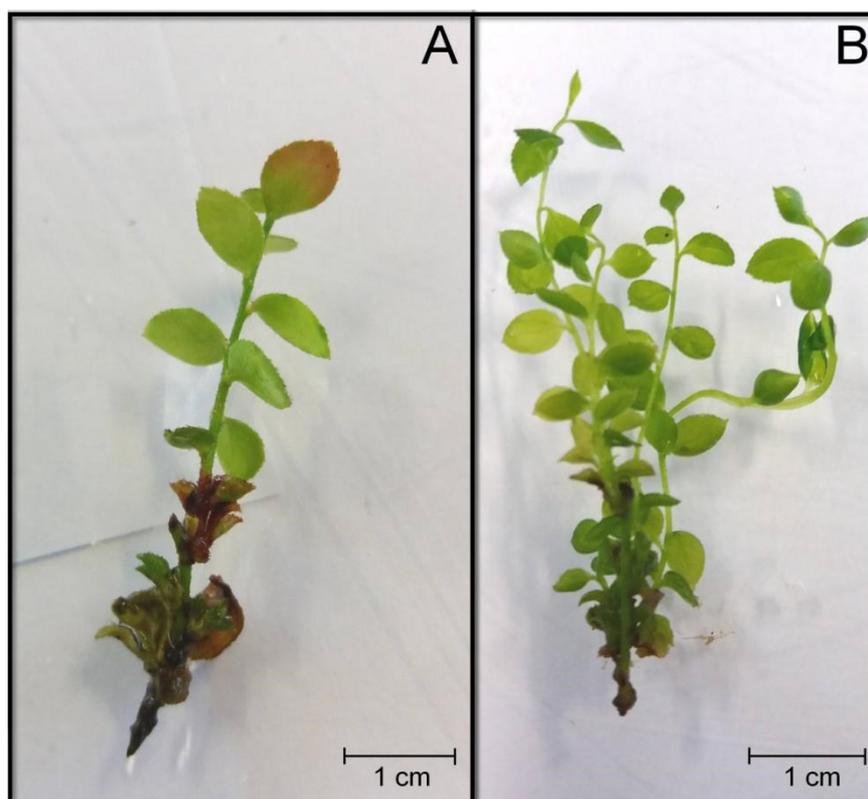


Figure 6. Blueberry healthy shoots obtained with 20 g L⁻¹ of sucrose (A) compared with hyperhydric shoots obtained with 10 g L⁻¹ sucrose (B) grown under TIS.

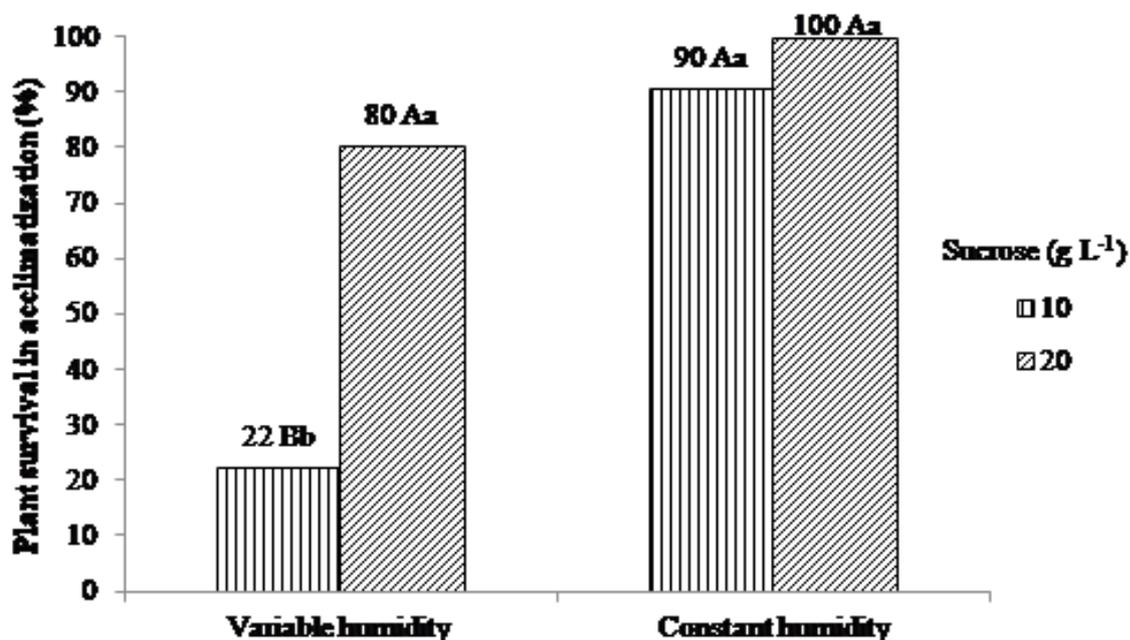


Figure 7. Influence of sucrose concentration on blueberry plant survival throughout acclimatization. Means followed by the same lowercase letters between sucrose concentration and the same uppercase letters between relative humidity do not differ statistically among treatments by the Tukey test ($p \leq 0.05$).

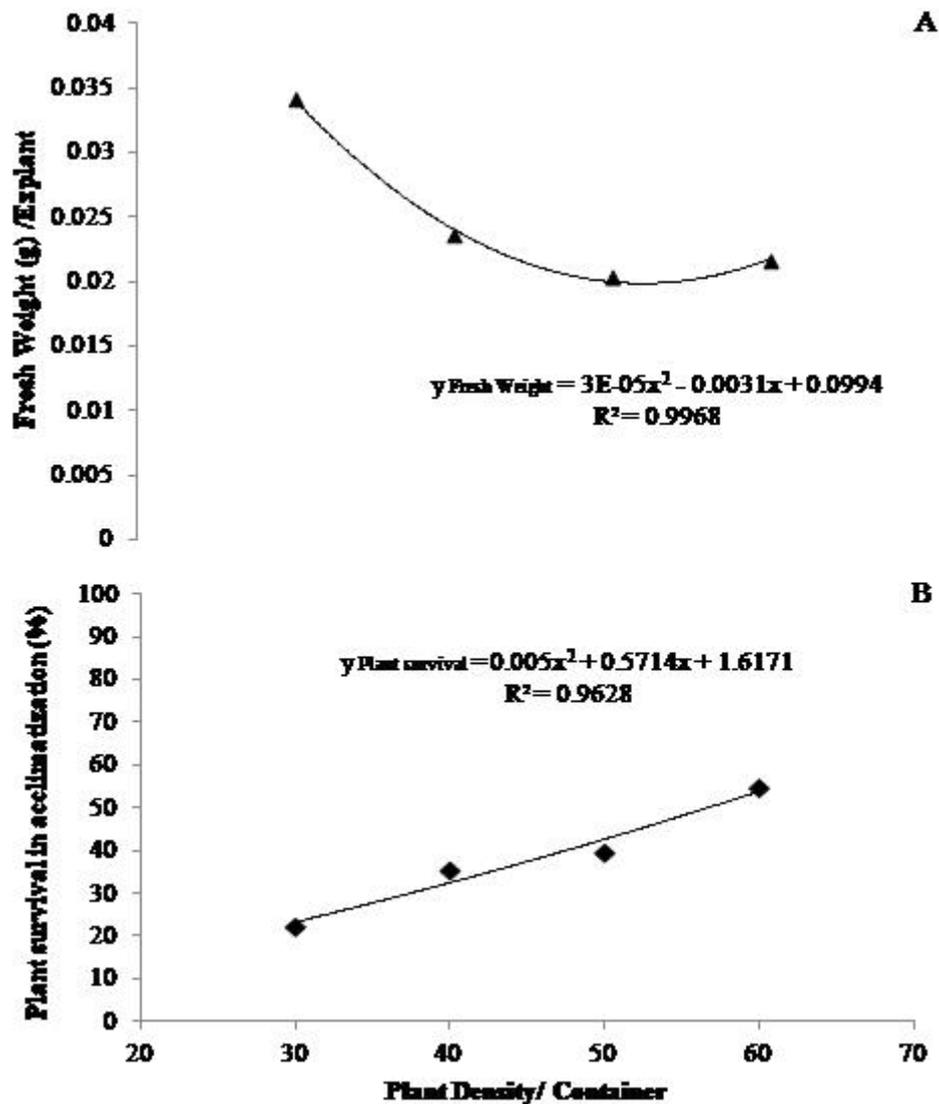


Figure 8. Plant density influence on fresh weight per explant under TIS (A) and plant survival through acclimatization (B).

The estimated value of 15 g L^{-1} considered to be the best sucrose concentration to be borne in mind was quite similar to that obtained by Cao et al. (2003) when it comes to micropropagation technique to be applied to *Vaccinium Corymbosum* L. cv. Duke. The 10 g L^{-1} sucrose concentration above providing the best averages in light of TIS was the one conducive to the lowest survival rates under acclimatization conditions. The usage of the liquid medium WPM might favor hyperhydration of plant tissues, galvanizing intensity of such physiological disorder.

It is well known in the literature that adequation of concentration of components, such as sucrose turns out to be pivotal to avoid hyperhydricity and also increase survival of plants throughout acclimatization, mainly because such a disturb seems to be linked to low *ex vitro* survival rates (ROJAS-MARTÍNEZ et al. 2010, DRIES et al. 2013, RAMÍREZ-MOSQUEDA et al. 2016). Hyperhydricity was also similarly reported by Tetsumura et al. (2008), who examined the effects of

different plant tissue culture types on micropropagation of *V. corymbosum* e *V. virgatum*, from which “Berkeley” cultivar when grown in MS mediums culminated in the highest incidence of hyperhydric shoots.

In view of such a problem, the more the production system approaches to the natural environmental conditions the better acclimatization of plants will be evidenced so that losses during critical phenological stage of the crop might be reduced. The hyperhydric plants are to be more sensitive to environmental stress and to rapid dehydration. Thus, under a small number of plants per container hyperhydricity itself impairs development and acclimatization of blueberry plantlets.

On the other hand, plants grown under a sucrose concentration corresponding to 20 g L⁻¹ showed the highest survival rates (80%) in conjunction with the best acclimatization possibly garnered. Our outcomes corroborate the findings reported by Debnath (2017), which highlighted that a survival rate ranging from 80 to 90% throughout acclimatization was obtained whenever plants were grown under TIS and stationary systems, respectively. Survival of plants is favored by means of TIS because considerably improves leaf thickness and also functionality of stomata (BRAGA, et al. 2010).

Conclusions

The 10 g L⁻¹ sucrose concentration clearly promoted formation of hyperhydricity, reducing in turn survival throughout acclimatization, whereas both the 20 g L⁻¹ coupled with 60 plants per container did favor survival rates. Further scientific investigations are supposed to be conducted in order to examine the impact of different plant density levels on blueberry micropropagation under TIS approach.

Acknowledgments

The authors are very grateful to Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Universidade Estadual de Ponta Grossa (UEPG) for the logistical and financial supports provided to the current research.

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